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Transcription of a Vaccinia Virus Late Promoter Template: Requirement for the Product of the A2L Intermediate-Stage Gene

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Evidence is presented that a 26-kDa protein encoded by the vaccinia virus A2L open reading frame, originally shown to be one of three intermediate-stage genes that together can transactivate late-stage gene expression in transfection assays (J. G. Keck, C. J. Baldick, and B. Moss, Cell 61:801–809, 1990), is required for in vitro transcription of a template with a late promoter. The critical step in this analysis was the preparation of an extract containing all the required factors except for the A2L protein. This extract was prepared from cells infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase in the presence of the DNA synthesis inhibitor cytosine arabinoside and transfected with plasmids containing the two other known transactivator genes, A1L and G8R, under T7 promoter control. Reaction mixtures made with extracts of these cells had background levels of late transcription activity, unless they were supplemented with extracts of cells transfected with the A2L gene. Active transcription mixtures were also made by mixing extracts from three sets of cells, each transfected with a gene (A1L, A2L, or G8R) encoding a separate factor, indicating the absence of any requirement for their coexpression. To minimize the possibility that the A2L protein functions indirectly by activating another viral or cellular protein, this gene was expressed in insect cells by using a baculovirus vector. The partially purified recombinant protein complemented the activity of A2L-deficient cell extracts. Recombinant A1L, A2L, and G8R proteins, all produced in insect cells, together complemented extracts from mammalian cells containing only viral early proteins, concordant with previous in vivo transfection data.

The large double-stranded DNA genome of vaccinia virus, the best characterized member of the poxvirus family, contains approximately 200 genes that are expressed in a programmed fashion (24). These genes have been divided into early, intermediate, and late classes on the basis of their time of expression and requirements for unique promoter sequences and *trans*-acting factors (23). The components needed for transcription of the early genes are packaged within the virion core and are brought into the cell during infection. The early mRNAs encode enzymes and factors that are needed for viral DNA replication and for transcription of the intermediate genes. The intermediate mRNAs are translated into factors needed for the transcription of late genes. Finally, the late mRNAs encode the early transcription system, which is packaged within the assembling virus particle and used for the next round of infection.

The factors required for early (1, 3, 5, 31, 32) and intermediate (29, 30, 34, 35) gene expression were found by biochemical fractionation procedures. In contrast, the late factors were identified primarily by a novel reverse genetic approach (17). Cells were (i) infected with vaccinia virus in the presence of cytosine arabinoside (araC) to prevent DNA replication and expression of intermediate genes encoding late *trans*-acting factors, (ii) transfected with a plasmid containing a reporter gene regulated by a late promoter, (iii) transfected with libraries of cloned vaccinia virus genomic fragments, and (iv) assayed for reporter gene expression. From this systematic screening, three intermediate genes (A1L, A2L, and G8R) were found to be necessary and sufficient for reporter gene expression in cells infected with vaccinia virus in the presence of araC.

Because the evidence that the A1L, A2L, and G8R gene products are required for late transcription was derived entirely from transfection data, the action of one or more of these proteins could have been indirect. For this reason, the proteins were called *trans*-acting factors rather than transcription factors (17). Subsequently, partially purified A1L and G8R proteins were shown to stimulate the transcription of a late promoter template in vitro, consistent with roles as transcription factors (19, 37, 39). Other components needed for late transcription in vitro include an early protein factor(s) (22, 37) and the viral RNA polymerase, both of which are synthesized in the presence of araC. Wright and Coroneos (37) reported that these four components (i.e., the A1L and G8R proteins, the early factor(s), and the RNA polymerase) are necessary and sufficient for in vitro transcription, leaving the specific role of the A2L protein in doubt and thereby raising a question regarding the earlier in vivo transfection studies of Keck et al. (17). Interpretation of the in vitro studies, however, was critically dependent on whether the four required components were free of contaminating A2L protein. To resolve this question, we modified the original transfection system (17) and used it to prepare extracts that did require exogenous A2L protein, as well as A1L and G8R proteins, for late transcription in vitro. This work, previously summarized (28) and presented here in detail, together with the recent report of Hubbs and Wright (14) firmly establish that the A2L gene product is a bona fide late transcription factor.

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MATERIALS AND METHODS

Cells and vaccinia virus. CV-1 cell monolayers were grown in Dulbecco's Modified Eagle Medium containing 0.03% L-glutamine and 10% fetal bovine

serum. CV-1 cells in 162-cm^2 cell culture flasks were left untreated or were treated for 2 h with 40 μg of araC per ml before being infected with a vaccinia virus recombinant, vTF7-3 (13), encoding the bacteriophage T7 RNA polymerase. Viral infections were carried out with 10 PFU per cell for 30 min at 37°C in the presence or absence of araC as indicated.

Baculoviruses were propagated in SF-9 cells (33), derived from the fall armyworm (*Spodoptera frugiperda*), in Grace's Insect Medium supplemented with 0.5 g of $CaCl_2$ per liter, 2.8 g of KCl per liter, 3.33 g of lactalbumin hydrolysate per liter, 3.3 g of yeastolate per liter, and 0.6 g of L-glutamine (GIBCO/BRL, Gaithersburg, Md.) per liter and containing 10% heat-inactivated fetal bovine serum. High Five BTI-TN-5B1-4 cells (Invitrogen, San Diego, Calif.), derived from the cabbage looper (*Trichoplusia ni*), were grown in complete supplemented Grace's Insect Medium as described above and used for the production and analyses of recombinant proteins. High Five cell monolayers in 162-cm^2 flasks were infected with 20 PFU per cell and rocked on a platform at room temperature. After 1 h, the virus inoculum was removed and replaced with complete fresh medium (26). Infected cells were incubated at 27°C and harvested at 48 h after infection.

Recombinant baculoviruses. Baculovirus vAc26N was constructed by homologous recombination using plasmid pAc26H, containing an N-terminal six-histidine-tagged A2L open reading frame, and linear DNA from *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) strain C6 (BaculoGold; PharMingen, San Diego, Calif.). Plasmid pAc26H is a derivative of pVL1392T7 (19, 36) in which the A2L open reading frame preceded by the bacteriophage T7 ϕ10 promoter (10) and the encephalomyocarditis virus untranslated leader sequence (15) was cloned at the *Eco*RV restriction site (3.1 map units) (26) upstream of the Ac*M*NPV polyhedrin gene promoter. Thus, this plasmid contains the A2L open reading frame regulated by the polyhedrin promoter in place of the polyhedrin gene, T7 promoter, and encephalomyocarditis virus leader upstream of the polyhedrin promoter to allow expression in the vaccinia virus T7 promoter-mediated expression system. The resulting recombinant baculovirus was selected on the basis of its plaque phenotype (occlusion negative) and shown to produce recombinant protein.

Baculoviruses expressing the vaccinia virus G8R gene, vAc30 (37), and the A1L gene with a six-histidine tag, vAc17N (19), were previously described.

Transfections and transient β-galactosidase assays. CV-1 cell monolayers in 162-cm^2 cell culture flasks were treated for 2 h with 40 μg of araC per ml and then infected with vTF7-3 in the presence of araC as described above. Transfections were performed after virus adsorption by the calcium phosphate precipitation method (11). Briefly, each 162-cm^2 flask was transfected with 60 μg of each plasmid DNA (2 μg of each plasmid per 10^6 cells), unless otherwise specified. Transfected plasmids containing the A1L, A2L, or G8R gene under T7 promoter control were previously described (19). Plasmid pUC18 was used as needed to maintain a constant concentration of DNA per transfection in each experiment. In addition, 37.5 μg of the reporter plasmid p11Xβ (1.25 μg/10^6 cells) (17) were used for transient-expression experiments. The reporter plasmid p11Xβ contains the promoter of the late 11K gene regulating the *Escherichia coli lacZ* gene. DNA was coprecipitated in polystyrene tubes by adding 3 ml of transfection buffer (0.14 M NaCl, 5 mM KCl, 1 mM $Na_2HPO_4 \cdot 2H_2O$, 0.1% dextrose, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.05]) and 150 μl of 2.5 M calcium chloride and incubating at room temperature for 20 min. The transfection mixture containing 40 μg of araC per ml was added to a monolayer of cells and incubated for 30 min at room temperature. After 30 min, 27 ml of complete medium was added and the incubation continued for 4 h at 37°C. Finally, the transfection mixture was removed, the monolayer was rinsed once with medium, and 25 ml of fresh complete medium was added.

A colorimetric assay was used to measure the β-galactosidase activities in cell lysates of transfected cells. Cells were washed with phosphate-buffered saline (pH 7.0) and lysed with reporter lysis buffer (Promega Corporation, Madison, Wis.), and β-galactosidase activity was determined by using the substrate *o*-nitrophenyl-β-D-galactopyranoside as specified by the supplier (Promega Corporation).

Extract preparation. Vaccinia virus-infected cells were harvested at approximately 22 h after infection (17 h after transfection), and baculovirus-infected cells were harvested at 48 h after infection by scraping the monolayers and centrifugation at 300 × *g* for 12 min. All subsequent procedures were performed at 4°C. Cell pellets were washed once with phosphate-buffered saline (pH 7.0 for CV-1 cells and pH 6.2 for *T. ni* cells) and resuspended in 5 volumes of buffer A (50 mM Tris-HCl [pH 7.9], 0.5 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF]), and cells were allowed to swell for 20 min on ice with frequent but gentle mixing. Swollen cells were spun at 300 × *g* for 15 min, and pellets were resuspended in 2 volumes of buffer B (5 mM Tris-HCl [pH 7.9], 0.42 M NaCl, 17% glycerol, 1 mM DTT, 1 mM MgCl, 1 mM PMSF) and Dounce homogenized by using 15 to 20 strokes. The homogenate was incubated on ice for 30 min with gentle mixing, and particulate material was sedimented at 9,300 × *g* for 20 min. The supernatant containing soluble cytoplasmic and nuclear proteins was allowed to precipitate for approximately 30 min after adding 0.35 g of solid ammonium sulfate per ml. Precipitated proteins were collected by centrifugation for 20 min at 9,300 × *g* and resuspended in 63 μl of buffer C (50 mM Tris-HCl [pH 7.9], 50 mM KCl, 1 mM DTT, 25% glycerol, 1 mM PMSF) unless stated otherwise. Finally, whole-cell extract proteins were dialyzed overnight against buffer C.

In vitro transcription assays. In vitro transcription reactions were performed with the G-less template pCFW9, which has the 11K late promoter, essentially as previously described (40). Various amounts of extracts diluted to 20 μl with 100 mM NaCl–50 mM Tris-HCl (pH 8.0)–0.1 mM EDTA–0.01% Nonidet P-40–2 mM DTT–10% glycerol were incubated on ice for 10 min before adding 1 μg of template. After incubating proteins and template for 10 min on ice, 20 μl of buffer containing 5 mM Tris-HCl (pH 8.0), 0.35 mM EDTA, 5 mM MgCl, 5 mM ATP, 0.5 mM CTP, 0.05 mM UTP, 4.8% polyvinyl alcohol, and 10 μCi of [α-^{32}P]UTP was added and the mixtures were incubated for 30 min at 30°C. Frequently, extracts from two or three separate transfections were mixed and the transcriptional activities were compared with those of extracts from one transfection. In these cases, the total amount of cell extract in each reaction mixture was kept constant so that the concentrations of RNA polymerase and early factor(s) were identical. However, reaction mixtures containing a mixture of two or three extracts may contain one-half or one-third, respectively, of factors expressed from transfected plasmids compared with reaction mixtures containing a single extract. The transcription products were resolved by polyacrylamide gel electrophoresis (PAGE), and radioactivity was detected by autoradiography or with a Betascope 603 blot analyzer (Betagen Corp., Waltham, Mass.). Figures were prepared by using Adobe Photoshop 3.

Chromatography of recombinant protein from baculovirus-infected cells. A whole-cell extract prepared from approximately 8 × 10^8 vAc26N-infected cells (53 mg of protein) in 250 mM NaCl–50 mM Tris-HCl (pH 8.0)–0.1 M EDTA–2 mM DTT–10% glycerol–0.5 mM PMSF was applied to a 5-ml DEAE-cellulose DE-52 column (Whatman, Fairfield, N.J.) equilibrated in the same buffer. The loaded column was washed with the same buffer, and unbound material was collected. Fractions containing the A2L protein (16.5 mg in 5.7 ml) were pooled and applied directly to a 3-ml hydroxylapatite Bio Gel HT column (Bio-Rad, Richmond, Calif.) equilibrated in 10 mM sodium phosphate (pH 6.8)–2 mM DTT–0.01% Nonidet P-40–10% glycerol. The sample was loaded onto the column, which was then washed with 10 column volumes of equilibration buffer. Proteins were eluted with 4 column volumes of the same buffer containing 0.1, 0.2, or 0.5 M sodium phosphate. Immunoblotting (see below) was used to detect the presence of the A2L protein, and Bio-Rad protein reagent was used to measure total protein.

Analysis of protein synthesis in insect cells. High Five cells were infected with wild-type Ac*M*NPV or vAc26N and pulse-labeled with 25 μCi of [^{35}S]methionine (Dupont NEN, Boston, Mass.) for 1 h before lysis (26). Proteins were resolved by sodium dodecyl sulfate (SDS)-PAGE and visualized by fluorography.

Immunoblotting. Proteins were electrophoretically separated on an SDS–12% polyacrylamide gel and transferred to a nylon membrane. After transfer, the membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 and proteins were reacted with a 1:1,000 dilution of antiserum raised against an A2L fusion protein made in *E. coli* (19). The membrane was washed with Tris-buffered saline containing Tween 20, and alkaline phosphatase-conjugated goat anti-rabbit antibody and nitroblue tetrazolium (NBT) were used to detect immunoreactive proteins.

RESULTS

Experimental strategy. Transfections of plasmids containing the vaccinia virus intermediate-stage genes A1L, A2L, and G8R were necessary and sufficient for the expression of a cotransfected reporter gene under late promoter control in araC-treated cells infected with vaccinia virus (17). If the A2L plasmid was omitted or if an A2L plasmid with an out-of-frame mutation was used, no reporter gene expression was detected. We considered, therefore, that extracts prepared from such cells should also require the protein product of the A2L gene for in vitro transcription of a late promoter-controlled G-less cassette template. In order to achieve high-level expression of viral gene products, we infected araC-treated CV-1 cells with vTF7-3 (13), a recombinant vaccinia virus containing the bacteriophage T7 RNA polymerase gene regulated by an early-late promoter, which permitted synthesis of the polymerase in the absence of DNA replication. Cells were then transfected with one or more plasmids encoding A1L, A2L, and G8R genes under T7 promoter control (Fig. 1). The infected cells were harvested 17 h later, and extracts were prepared for transcription assays. Initially, we also transfected a sample of cells with the reporter plasmid p11Xβ, which contains a late promoter regulating the *E. coli lacZ* gene in order to monitor infection and transfection efficiencies, ensure the adequacy of the araC block, and confirm that A1L, A2L, and G8R were necessary and sufficient for reporter gene expression. Once the

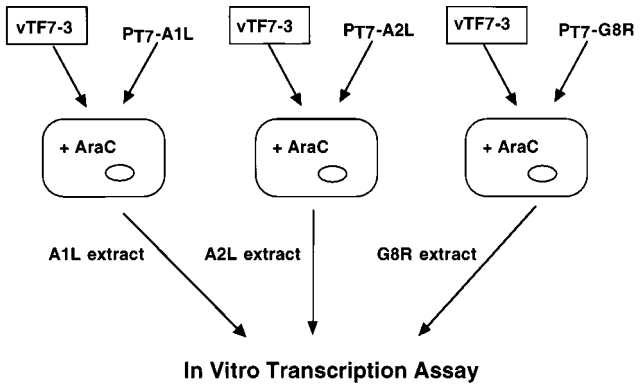

FIG. 1. Assay for the in vitro transcriptional activities of proteins encoded by transfected genes. Mammalian cells, treated with the DNA replication inhibitor araC, are infected with a recombinant vaccinia virus, vTF7-3, expressing the T7 RNA polymerase gene. Cells are then transfected with an individual plasmid or combinations of plasmids containing the A1L, A2L, and/or G8R open reading frames under T7 promoter (P_{T7}) control. Cells are harvested, and extracts are processed and tested separately or in combination for the ability to transcribe a vaccinia virus late promoter template.

method had been optimized, the reporter plasmid was omitted from the transfection protocol.

Late transcription activity of extracts from transfected cells. Extracts prepared from cells infected with vaccinia virus in the absence of araC and containing all the factors necessary for late gene expression were used to establish transcription conditions (Fig. 2A, lanes 1 and 2). Similar extracts from cells infected with vaccinia virus in the presence of araC were not competent to support late gene transcription because DNA replication and consequently intermediate gene expression were blocked (Fig. 2A, lanes 3 and 4). However, extracts of araC-treated infected cells transfected with three plasmids containing the A1L, A2L, and G8R open reading frames were able to reconstitute late gene transcription (Fig. 2A, lanes 5 and 6).

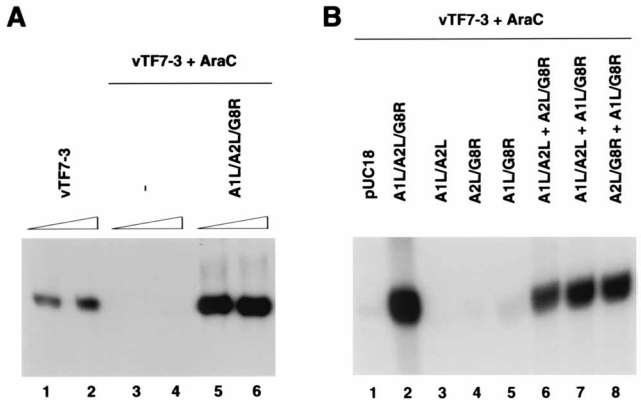

FIG. 2. Transcription of a late promoter template with extracts containing the A1L, A2L, and G8R proteins. (A) In vitro transcription with extracts from infected cells either untreated (lanes 1 and 2) or treated (lanes 3 to 6) with araC and transfected with plasmids encoding the A1L, A2L, and G8R proteins (lanes 5 and 6). The triangles above lanes denote the increasing volumes (5 and 10 μl) of extracts used in each reaction pair. (B) Cells were infected with vTF7-3 in the presence of AraC and transfected with pUC18 or simultaneously with two plasmids encoding the A1L, A2L, or G8R gene. Plasmids with gene designations separated by a shill were cotransfected; a plus sign denotes the use of more than one extract. Extracts from one transfection (9 μl; lanes 1 to 5) or two transfections (4.5 μl each; lanes 6 to 8) were used in transcription assays.

Specific transcription factor requirement. We next determined the effect(s) of omitting one of the three intermediate transactivators. Vaccinia virus-infected cells were transfected with two of the three plasmids encoding the A1L, A2L, and G8R proteins in the presence of araC. The omission of any one of these three plasmids reduced transcription to near background levels (Fig. 2B; compare lane 2 with lanes 3 to 5), indicating that all three proteins were needed to stimulate late transcription in vitro as well as in vivo.

Further studies showed that transcription obtained by mixing two independent extracts, each containing two of these three transcription factors, approached that of transcription with an extract containing all three proteins (Fig. 2B, lanes 6 to 8). The slightly lower levels of transcription in reconstituted reactions (Fig. 2B; compare lane 2 with lanes 6 to 8) may be explained by the dilution of factors when two or more extracts were mixed (see Materials and Methods). Importantly, similar levels of transcription activity were obtained regardless of which two plasmids were transfected into the same cell, suggesting that the activities were independent of each other. We did notice that transcription reactions sometimes had a slightly higher background when the A2L gene was omitted from transfections than when the A1L or G8R gene was omitted (Fig. 2B, lanes 3 to 5). However, a similar phenomenon was noted with in vivo reporter gene assays (data not shown). It is possible that intermediate genes are transcribed at a very low level in the presence of araC and that trace amounts of the A2L product have a detectable effect. The other intermediate genes may be less leaky, or their products may be required in larger amounts.

To rule out the possibility that the A1L, A2L, and G8R genes need to be coexpressed for activity and to evaluate the influence of each gene product in transcription, we performed additional transfections. First, we demonstrated that extracts prepared from cells transfected with a single gene could complement extracts from cells transfected with a combination of the other two genes. This was true for all three intermediate genes (Fig. 3, lanes 5, 9, and 13). To overcome the dilution of transfection factors when two extracts were mixed, we also transfected cells with increased amounts of the A2L plasmid. Thus, extracts of cells transfected with three times the standard amount of the A2L gene could stimulate in vitro transcription of extracts from cells transfected with the A1L and G8R genes to the same level as that in extracts from cells transfected with all three genes (Fig. 3; compare lanes 2 and 10).

Finally, when extracts of cells transfected with single genes were mixed and assayed, there was significant (seven times the background) transcriptional activity (Fig. 3; compare lanes 4, 7, 12, and 14). The dilution effect of mixing extracts was partially overcome by transfecting larger amounts of the A2L plasmid, resulting in still higher activity (Fig. 3, lane 15). Under the latter conditions, extracts had approximately half of the transcriptional activity of those from cells cotransfected with all three plasmids (Fig. 3; compare lanes 2 and 15). Because the products of the A1L and G8R genes were also diluted by mixing extracts and we did not compensate for this by transfecting additional DNA, it is not surprising that this activity was not equivalent to that obtained by cotransfecting these three genes.

Expression of the A2L gene in baculovirus-infected cells. The results obtained by mixing extracts of separate transfections demonstrated that in vitro transcription activity could be obtained even if the products of the A1L, A2L, and G8R genes had no opportunity to interact in vivo. Nevertheless, the possibility that the A2L protein activated or modified a still unknown viral or cellular factor prior to the lysis of araC-treated

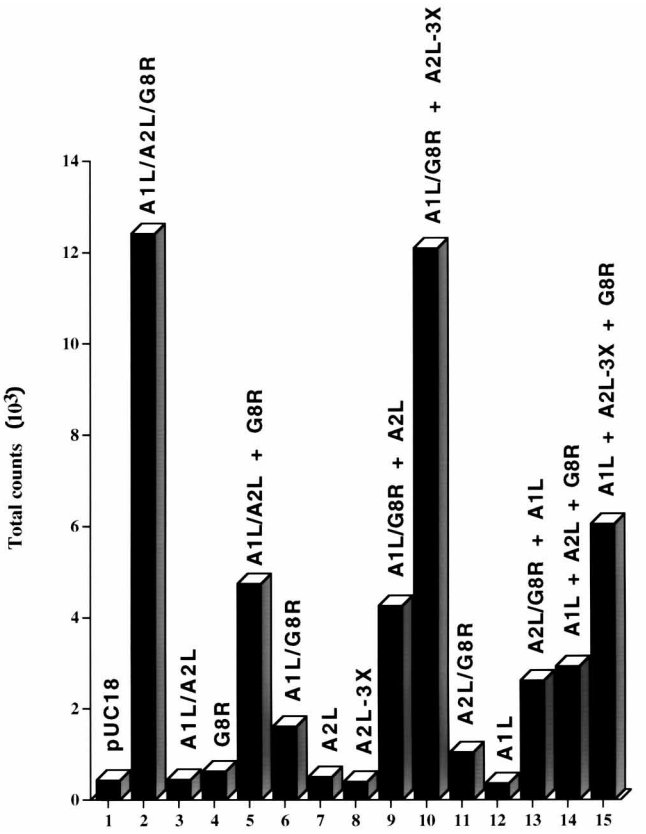

FIG. 3. Requirements of the A1L, A2L, and G8R gene products for in vitro transcription. Cells were infected with vTF7-3 in the presence of araC and transfected with the plasmids indicated above each bar. Plasmids with gene designations separated by a shill were cotransfected; a plus sign denotes the use of more than one extract. Transfections with three times the amount of the A2L plasmid are designated A2L-3X. All of the transcription reaction mixtures contained 9 μl of extract(s). In lanes 1 to 4, 6 to 8, 11, and 12, 9 μl of a single extract was used; in lanes 5, 9, 10, and 13, 4.5 μl each of two extracts was used; and in lanes 14 and 15, 3 μl each of three extracts was used.

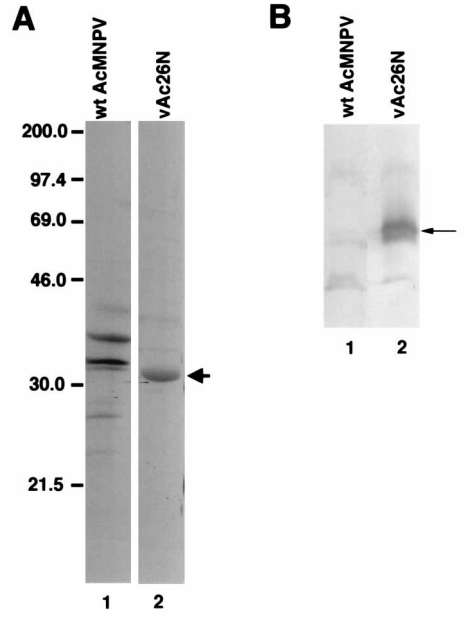

FIG. 4. Synthesis of A2L protein in insect cells by a recombinant baculovirus. (A) SDS-PAGE of proteins pulse-labeled with [^{35}S]methionine at 48 h after infection of High Five cells with wild-type Ac*M*NPV (wt AcMNPV; lane 1) or recombinant vAc26N (lane 2). The arrow on the right points to the position of recombinant A2L protein, which migrated to approximately 30 kDa in lane 2. A fluorograph is shown, with the positions of markers (in kilodaltons) on the left. (B) Immunoblot of proteins from insect cells infected with Ac*M*NPV (lane 1) or vAc26N (lane 2) and incubated with anti-A2L serum. The arrow on the right points to the position of the A2L product, which migrated to 30 kDa in lane 2.

infected cells remained. To address this possibility, we expressed the A2L gene in a heterologous system. The A2L gene, modified to encode a 26-kDa product with a six-histidine tag at its N terminus, was inserted into a baculovirus transfer vector. The vector also had a T7 promoter upstream of the baculovirus promoter, allowing us to verify that the product was biologically active when transfected into araC-treated vaccinia virus-infected cells by both reporter gene (data not shown) and in vitro assays. Recombination into the polyhedrin locus within the baculovirus genome was confirmed by plaque phenotype (data not shown), and expression of the recombinant protein was determined by metabolic labeling of infected cells. At 48 h after infection, a major labeled 26-kDa protein (Fig. 4A, lane 2) was detected by SDS-PAGE of recombinant vAc26N-infected cell lysates in place of the 33-kDa polyhedrin protein (Fig. 4A, lane 1) expressed by the Ac*M*NPV vector. The 26-kDa protein was not detected when metabolic labeling was performed at 3 h after infection, consistent with the use of the very late polyhedrin promoter (data not shown).

Immunoblotting with antiserum against an A2L fusion protein made in *E. coli* confirmed the identity of the recombinant 26-kDa protein. No major proteins in wild-type Ac*M*NPV cross-reacted with the antiserum, while one specific protein in vAc26N of the expected size reacted (Fig. 4B, lane 2). Thus, the Ac*M*NPV-produced recombinant protein was synthesized at the expected times during baculovirus infection, had the correct size, and reacted with specific antiserum.

Transcription factor activity of baculovirus-produced A2L protein. We next compared the in vitro activity of the Ac*M*NPV recombinant protein made in insect cells with that of the authentic vaccinia virus protein made in mammalian cells. Whole-cell extracts from insect cells infected with wild-type Ac*M*NPV or vAc26N were mixed with an extract from CV-1 cells treated with araC, infected with vTF7-3, and transfected with the A1L and G8R plasmids. The vAc26N-infected insect cell extract was able to complement the CV-1 cell extract for in vitro transcription, whereas the extract from Ac*M*NPV-infected insect cells could not (Fig. 5A, lanes 3 to 6). Moreover, the transcription factor activity of the recombinant A2L protein from the vAc26N extract was comparable to that of the authentic A2L protein from CV-1-infected and -transfected cells (Fig. 5A; compare lane 2 with lane 6). Further analyses indicated that the ability of the vAc26N extract to stimulate transcription was specific (i.e., required the products of the A1L and G8R genes) and was comparable in activity to that of extracts from insect cells infected with recombinant baculoviruses that express the A1L and G8R genes (Fig. 5B). In addition, transcription activity was obtained when extracts of araC-treated cells infected with vaccinia virus were supplemented with A1L, A2L, and G8R proteins produced in insect cells (Fig. 5C).

Chromatography of the recombinant A2L protein. The recombinant 26-kDa protein was prepared by ammonium sulfate precipitation and DEAE-cellulose and hydroxylapatite chromatography. Fractions were analyzed by immunoblotting and for transcription factor activity. The majority of immunoreactive protein and transcription factor activity eluted from the

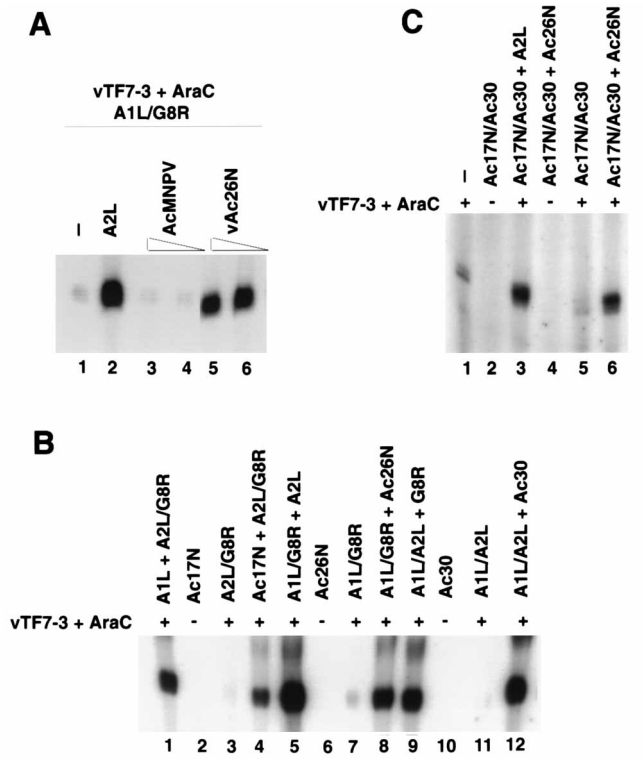

FIG. 5. Activities of baculovirus-produced late transcription factors. (A) A total of 4 μl of extracts from CV-1 cells infected with vTF7-3 in the presence of araC and transfected with plasmids containing the A1L and G8R genes was tested alone (−; lane 1) or mixed with 4 μl of an extract from araC-treated, vTF7-3-infected CV-1 cells transfected with the A2L gene (lane 2) or an extract (1 or 0.5 μl) from insect cells infected with wild-type Ac*M*NPV (lanes 3 and 4) or vAc26N (lanes 5 and 6). The triangles above lanes denote decreasing amounts of the indicated extracts. Transcription products were analyzed by PAGE, and an autoradiogram is shown. (B and C) Components of the transcription reaction are indicated above the lanes of each autoradiogram. A plus or minus sign indicates the presence or absence, respectively, of extract derived from CV-1 cells infected with vTF7-3 in the presence of araC (4 μl); A1L, A2L, and G8R refer to plasmids transfected or cotransfected (shill) into the aforementioned CV-1 cells; Ac17N, Ac30, and Ac26N, refer to extracts of insect cells infected with baculoviruses encoding G8R, A1L, and A2L, respectively, with a shill indicating doubly infected cells. A plus sign between extract designations indicates that both were added to the same reaction mixture. An equivalent of either 0.5 (B) or 0.3 (C) μl of each insect whole-cell extract was used in each reaction.

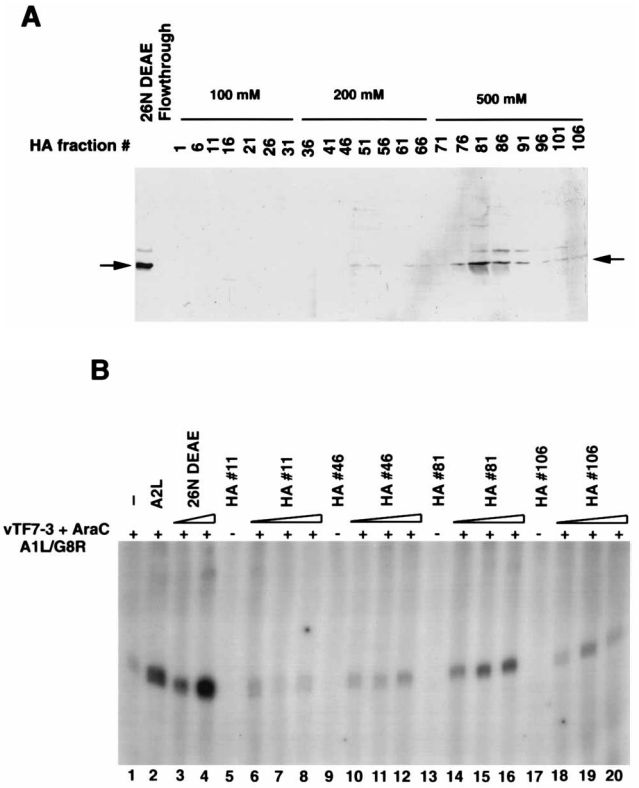

FIG. 6. Transcriptional activity of hydroxylapatite-chromatographed recombinant A2L protein. An extract of vAc26N-infected cells was prepared by ammonium sulfate precipitation and chromatography on DEAE-cellulose and hydroxylapatite columns. (A) SDS-PAGE immunoblot of pooled DEAE fractions (26N DEAE), hydroxylapatite (HA) flowthrough, and numbered fractions eluting with 100, 200, or 500 mM sodium phosphate step gradient. The position of the A2L protein is indicated by arrows on the left and right. (B) Transcription reaction mixtures containing 6 μl of extracts from araC-treated CV-1 cells infected with vTF7-3 and transfected with plasmids containing the A1L and G8R genes (A1L/G8R) are indicated by a plus sign above the lane. The components of other extracts added to reaction mixtures are also indicated. Lanes: 2, A2L, extract derived from araC-treated CV-1 cells infected with vTF7-3 and transfected with an A2L plasmid; 3 and 4, 26N DEAE, extract from vAc26N-infected cells passed through DEAE-cellulose column; 5 to 20, HA fractions. The triangles above lanes indicate that increasing amounts of 26N DEAE (0.2 or 1 μl) or HA fractions (1, 3, or 6 μl) were used. The reaction mixtures for lanes 1, 5, 9, 13, and 17 contained 6 μl of the designated extracts.

hydroxylapatite column with 0.5 M sodium phosphate buffer (Fig. 6). As expected, transcription was also dependent on the A1L and G8R gene products. Although this recombinant protein was engineered to contain an N-terminal six-histidine tag, it did not bind avidly to an affinity column (data not shown).

DISCUSSION

The present data, together with the recent report of Hubbs and Wright (14), indicate that the A2L gene is a bona fide transcription factor, confirming the predictions of Keck et al. (17) regarding the requirement for the three intermediate genes, A1L, A2L, and G8R. The vaccinia virus late transcription factors encoded by these genes may be referred to as VLTF-1 (G8R), VLTF-2 (A1L), and VLTF-3 (A2L). The key step in this study was the preparation of an extract containing all the required factors except for the A2L protein. This task was accomplished in a facile and reproducible way that could be correlated with previous in vivo studies by (i) infecting cells with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase in the presence of the DNA synthesis inhibitor araC and (ii) transfecting these cells with plasmids containing the two other known transactivator genes, A1L and G8R, under T7 promoter control. Extracts of these cells had background levels of late transcription activity, unless they were supplemented with extracts of cells transfected with the A2L gene. Active transcription mixtures were made by mixing extracts from three sets of cells, each transfected with a gene (A1L, A2L, or G8R) encoding a separate factor, indicating the absence of any requirement for their coexpression. To minimize the possibility that the A2L protein functions indirectly by activating another viral or cellular protein in vivo, the gene was expressed in insect cells by using a baculovirus vector. The recombinant protein complemented the activity of A2L-deficient cell extracts. Furthermore, recombinant A1L, A2L, and G8R proteins, all produced in insect cells, together complemented extracts from mammalian cells infected with vaccinia virus in the presence of araC.

Conditional lethal temperature-sensitive A1L (6) and inducer-dependent G8R (42) mutants show defects in late gene

expression, establishing the roles of these proteins during the virus replication cycle. No conditional lethal mutant of the A2L gene has yet been described.

Many eukaryotic transcription factors possess zinc finger motifs of either the C_2H_2 or C_2C_2 type (7, 27). A2L contains an amino acid sequence, $CX_2CX_{13}CX_2C$, that resembles the C_2C_2 type, and a recombinant fusion protein was shown to bind zinc in vitro (18). Conservative cysteine-to-serine mutations within this motif prevented zinc binding and also inactivated the transactivating function of A2L in transfection assays. Typical zinc finger motifs are not present within the A1L or G8R protein, although the former has a $CX_2CX_{23}CX_3C$ sequence, with uncommon spacing between the second pair of cysteines, and has been shown to bind zinc in vitro (18). It will be of interest to study the protein-protein and protein-DNA interactions of these transcription factors.

The early components present in cells infected with vaccinia virus in the presence of araC that are required for late transcription include the viral multisubunit RNA polymerase (25) and one or two additional proteins called P3 factor (22) and VLTF-X (14). The polypeptides comprising the vaccinia virus RNA polymerase are encoded by eight genes, all of which are expressed early and late in infection. RAP94, a 94-kDa polypeptide that is tightly associated with RNA polymerase packaged in virions, is encoded by the H4 gene, which is expressed only at late times after DNA replication (2, 16). RAP94 is required for the transcription of early genes (1, 2, 8) but cannot be required for the transcription of either intermediate or late genes, because RNA polymerase made in the presence of araC is functional both in vivo (17) and in vitro (22). Direct evidence to support this view was obtained by Zhang et al. (41), who specifically repressed the H4 gene by using the *E. coli lac* operator system and demonstrated that the expression of both intermediate and late genes occurred normally in the absence of this protein. Furthermore, extracts lacking RAP94 and unable to transcribe early promoter templates were more transcriptionally active on late promoters than were extracts from wild-type virus-infected cells, raising the possibility that RNA polymerase containing RAP94 is not functional or less functional than is RNA polymerase lacking RAP94 (41). By chromatographic separation methods, Wright and Coroneos (38) arrived at similar conclusions.

The finding that at least one of the late transcription factors is expressed early in infection, rather than at intermediate times, was surprising, as it deviates from a strict cascade model in which factors are made in the immediately preceding temporal stage (22, 37). One factor, P3 (22), was recently identified as the product of the early-late H5R gene, expressed in an active form in insect cells, and given the name VLTF-4 (20, 21). The VTLF-X factor described by Wright and Coroneos (37) eluted from phosphocellulose at a lower salt concentration than did P3, so we do not know whether it is the H5R gene product or another protein. Still other late transcription factors might be discovered by using other templates or other assay conditions. Studies with conditional lethal mutants suggest that the A18R (4) and D11L (NPH I) (9) genes also have roles in late transcription.

The vaccinia virus-bacteriophage T7 transient-transfection system has been widely used to express proteins for in vivo and in vitro studies. The innovation here was to prepare extracts from cells treated with araC, so that only viral intermediate and late genes delivered by transfection could be expressed. Such genes can be easily manipulated to have mutated coding sequences and used for studying protein-protein interactions or structure-function relationships in the absence of the endogenous wild-type protein. For individual experiments in this study, we routinely transfected 3×10^7 to 3×10^8 cells by the calcium phosphate technique. Although liposome-mediated transfections may be more efficient, the commercially available materials are too expensive for large-scale work. The relatively high levels of expression in our transfection system can be attributed to the use of vaccinia virus vectors expressing the T7 RNA polymerase gene and the encephalomyocarditis virus leader for cap-independent translation (12, 13).

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